Structural Analysis of Pick's Disease-Derived and *in Vitro*-Assembled Tau Filaments

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Pick's and Alzheimer's diseases are distinct neurodegenerative disorders both characterized in part by the presence of intracellular filamentous tau protein inclusions. The tight bundles of paired helical filaments (PHFs) of tau protein found in Alzheimer's disease (AD) differ from the tau filaments of Pick's disease in their morphology, distribution, and pathological structure as identified by silver impregnation. The filaments of Pick's disease are loosely arranged in pathognomonic spherical inclusions found in ballooned neurons, whereas the tau pathology of AD is classically described as a triad of neuropil threads, neurofibrillary tangles, and dystrophic neurites surrounding and invading plaques. In this study we used the high-resolution technique of scanning transmission electron microscopy to characterize and compare the filaments found in Pick's disease with those found in AD. In addition, we determined the mass/nm length and density of arachidonic acid-induced *in vitro*-assembled filaments. Three morphologically distinct populations of Pick's filaments were identified but each was indistinguishable from AD-PHF in mass/nm length and density. Filaments assembled *in vitro* from single isoforms were similar in mass/nm length, but less dense than AD-PHF and Pick's disease filaments. Finally, we provide clear structural evidence that a PHF, whether found in disease or assembled *in vitro*, is composed of two distinct intertwined filaments. (*Am J Pathol* 2001, 158:1481-1490)

Polymeric tau protein inclusions occur in a number of neurodegenerative diseases of aging. Filaments of tau protein constitute the fibrillar pathologies of Alzheimer's disease (AD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), frontotemporal dementia (FTDP-17), and Pick's disease. The distribution of tau

pathology across this spectrum of disorders is widespread, appearing in both neurons and glia, and throughout all cortical regions, cerebellum, and brainstem.¹⁻⁴

Each disease displays characteristic types of tau deposition targeting both unique and overlapping vulnerable cell populations. AD, the most common dementia of the aged, contains tau pathology typified by neurofibrillary tangles, neuropil threads, and dystrophic neurites surrounding and invading the A β containing plaques.⁵ These tau deposits are composed of filaments that are mostly paired helical (PHFs) in appearance, with a width of 10 to 20 nm and a periodicity of 80 nm; also present are a variable percentage of straight filaments (SFs) that are ~15 nm in width^{6,7} and predominate in neuropil threads.⁸ By contrast, Pick's disease is quite rare, representing only 0.4 to 2% of all dementias⁹ and it is characterized by the presence of ballooned neurons and argyrophilic Pick bodies, found mostly in frontal cortex and the dentate gyrus of the hippocampus, regions less affected in AD.¹

The Pick body itself has been described as a nonmembrane delimited spherical structure with a loose arrangement of tau filaments when compared to the compact bundles of tau filaments found within a neurofibrillary tangle.¹⁰ The tau filaments found within the Pick body are mostly SFs with a 15-nm width, but occasional PHF-like filaments with a long periodicity of 120 to 160 nm are also observed.¹⁰⁻¹³ As with many other frontal lobar atrophies (often termed "tauopathies"), extracellular deposits of A β are absent in Pick's disease.¹⁴

Alternative splicing of tau mRNA leads to the expression of six protein isoforms of tau in the adult CNS that differ in their expression of exons 2, 3, and 10.^{15,16} The SFs and PHFs found in different tauopathies are characterized by distinct mixtures of tau isoforms. PHFs of AD are comprised of all six tau isoforms,¹⁷ whereas other tauopathies such as PSP, CBD, and FTDP-17, form filaments comprised primarily of isoforms containing four microtubule binding repeats (4Rtau).¹⁸ Both intronic and exonic mutations in the tau gene have been described for FTDP-17, many of which affect the splicing of tau

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mRNA.^{19–21} This leads to an overexpression of exon 10, resulting in an increased ratio of 4R:3R isoforms.^{19–23}

In contrast to these findings, a marked decrease in 4R tau has been described in Pick's disease brain homogenates, suggesting that, unlike other filaments, Pick's filaments are comprised primarily of 3R tau.^{18,24,25} However, a recently discovered point mutation (G389R) in the tau gene apparently results in Pick's disease exhibiting tau deposits containing both 3R and 4R tau.²⁶ To determine the definitive composition of Pick's filaments, tau polymer-enriched fractions isolated from well-characterized Pick's disease cases were investigated. Moreover, the structure of these Pick's filaments was compared to that of AD-PHF's and filaments assembled *in vitro* from individual recombinant tau isoforms.

Materials and Methods

Cases

Autopsy brain tissue from the patients with AD (72-year-old female, 74-year-old female, and an 82-year-old male), and sporadic Pick's disease (63-year-old male, 89-year-old female, 69-year-old female, and a 72-year-old male) were provided by the Rush Presbyterian St. Luke's Alzheimer's Disease Center, the Albert Einstein College of Medicine (AECOM) Brain Bank, and the Cognitive Neurology and Alzheimer's Disease Center of Northwestern University Medical School. All AD cases met The Consortium to Establish a Registry for Alzheimer's Disease criteria for a diagnosis of probable AD (CERAD).^{27–30} The diagnosis of Pick's disease was based on the presence of neuronal Pick bodies and atrophy in the frontal and temporal lobes.³¹ Human fetal brain tissue was obtained from elective pregnancy terminations (19 to 24 weeks of gestation) through a protocol approved by the Committee on Clinical Investigations at AECOM.

Antibodies

Monoclonal tau antibodies (Tau 1, Tau 14, and Tau 46.1)^{32,33} were purified and handled as previously described;³⁴ the cell lines producing antibodies Tau 14 and Tau 46.1 were gifts from Dr. Virginia Lee, University of Pennsylvania Medical School. The PHF-1 monoclonal antibody³⁵ was the generous gift of Dr. Peter Davies of AECOM. Antibodies AT8 and AT100³⁶ were purchased from Endogen Inc. (Woburn, MA). The exon-specific polyclonal antibodies E2, E3, and E10²⁵ were the generous gift of Dr. Andre Delacourte (Inserm, Lille, France).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Proteins were separated by SDS-PAGE using 10% polyacrylamide gels and then transferred onto nitrocellulose paper. The blots were incubated with 5% nonfat dry milk

in Tris-buffered saline and then with primary antibodies for 2 hours at room temperature or overnight at 4°C. The secondary antibodies were conjugated to horseradish peroxidase. The specific antibody signals were detected using chemiluminescence reagents (Amersham Pharmacia, Piscataway, NJ). Filament fractions were dephosphorylated using a protocol modified from Yang and colleagues.³⁷ Briefly, filaments were treated with 4 mol/L guanidine for 1 hour, and then 15 IU/ml of alkaline phosphatase for 2 hours at 67°C in a buffer containing 50 mmol/L Tris/HCl, pH 8.0, 1 mmol/L dithiothreitol, and 1 mmol/L phenylmethyl sulfonyl fluoride.

Immunolabeling

For immunohistochemistry, free-floating 40- μ m sections were processed as previously described,³⁸ with monoclonal antibodies Tau 14 (1:2,000) and Tau 46.1 (1:1,000) overnight at room temperature. For immunoelectron microscopy, filaments were labeled as described earlier³⁹ using secondary antibodies conjugated to 10-nm colloidal gold particles (Amersham Pharmacia, Piscataway, NJ). Samples on grids were examined using a JEOL 100CX electron microscope at 80 kV.

Isolation of Tau Protein and Filaments

Sarkosyl insoluble filaments were purified as enriched fractions from Alzheimer's and Pick's disease brains as previously described.⁴⁰ Fetal tau was purified from human fetal brain⁴¹ and bovine tau from whole calf brain.⁴² Recombinant tau isoforms were expressed in the pT7C vector and purified as specified previously.⁴³ Filaments were assembled *in vitro* using 4 μ mol/L of tau protein in the presence of 75 μ mol/L of arachidonic acid (Cayman Chemical Company, Ann Arbor, MI) and 5 mmol/L dithiothreitol⁴³ for 24 to 30 hours at 25°C.

Transmission Electron Microscopy (TEM) and Scanning Transmission Electron Microscopy (STEM)

For TEM, *in vitro* polymerized tau samples were fixed in 2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA), and inverted over formvar/carbon-coated copper grids (Electron Microscopy Sciences). Grids were stained with 2% uranyl acetate and viewed using a JEOL JEM 1220 electron microscope operating at 60 kV. For STEM, samples were applied to grids as previously described.⁴⁰ STEM images were analyzed using PCMASS12 software, created by Dr. Joseph Wall. The mass/nm length of filament segments was calculated in reference to tobacco mosaic virus (131 kd/nm length), which served as an internal control on all STEM grids. The density of filaments was calculated using the following equation:⁴⁴

$$\text{Density} = \frac{\text{Mass per unit length (kd/nm)}}{\text{Width (nm)} \times \text{thickness (nm)}}$$

Figure 1. Monoclonal antibodies Tau 14 (A and C) and Tau 46.1 (B and D) were used to immunolabel the pathology seen in superior temporal gyrus of AD (A and B) and the frontal cortex of Pick's disease (C and D). Scale bar, 50 μ m.

For the purpose of this estimation of density, the width and thickness of filaments were considered to be the same. The width and periodicity of filaments was measured using Metamorph 4.0 software (Universal Imaging Corporation, West Chester, PA) and calibrated against 200-nm size nanosphere beads (Duke Scientific Corp., Palo Alto, CA).

Results

Tau Pathology in AD and Pick's Disease

Adjacent brain sections from selected regions of AD (superior temporal gyrus) and Pick's disease (frontal cortex) were immunostained for tau using Tau 14 and Tau 46.1 antibodies. In AD, typical tau-positive inclusions consisted of neurofibrillary tangles, neuropil threads, and dystrophic neurites surrounding and invading plaques (Figure 1, A and B). In Pick's disease, numerous Pick bodies were found that were labeled with both antibodies (Figure 1, C and D). In addition, there was a distinct neuropil pathology consisting of grain-like deposits detected with Tau 46.1 but not Tau 14. The neuropathology of Pick's cases was similar to that described previously.⁴⁵

Absence of Tau 46.1 staining in some but not all tau inclusions suggests that either the epitope is inaccessible or absent in some Pick bodies.

Ultrastructure of Pick's Filaments

When viewed by electron microscopy, filaments purified from Pick's disease brain exhibited both straight morphology (width, ~ 10 nm) (Figure 2A), and PHF-like morphologies (Figure 2, B and C). The majority of the PHF-like filaments displayed a longer periodicity (Figure 2B) than AD-PHF, whereas a small population of filaments showed a periodicity similar to that in AD-PHF (compare Figure 2, C and D). Measurements of periodicities identified a single peak with an average of ~ 80 nm along the long axis of the filament for AD-PHF, (Figure 2E, inset), and two distinct peaks for Pick's disease filaments (Figure 2E). The first peak was similar to that of AD-PHF, with an average period of ~ 90 nm; the second peak averaged ~ 145 nm, in agreement with previous findings.¹⁸ Filaments exhibiting the 90 nm axial periodicity were usually much shorter in length than those with the longer period, and have not been previously described in Pick's disease.

Figure 2. Electron micrographs of filaments from Pick's disease (A, B, and C) and AD (D) stained with uranyl acetate. The periodicity of twisted filaments was measured as in Materials and Methods, and the distribution of the pitch for paired filaments found in (E) Pick's disease and AD (inset) is shown. The average period for AD-PHF's was 76.1 ± 11.9 nm, Peak 1, average 91.3 ± 15.0 nm, peak 2, average 144.4 ± 30.7 nm. Scale bar, 100 nm. JEOL 100CX.

Pick's Filaments Contain Abnormally Phosphorylated Tau

Immunoelectron microscopy was used to verify the antigenic character of the filaments from Pick's disease. Pick's filaments were labeled with phospho-specific tau monoclonal antibody PHF-1 (and AT100, data not shown) in a manner similar to that of AD-PHF's (Figure 3, A and

B.), and also with the tau monoclonal antibodies Tau 14 and Tau 46.1 (Figure 3, C and D). When filament-enriched fractions from Pick's disease and AD were subjected to SDS-PAGE and Western blotting, different isoform patterns were observed. In AD, three characteristic bands of apparent molecular weights 60 kd, 64 kd, and 68 kd were immunoreactive to PHF-1 (Figure 4A). In contrast, Pick's disease filaments contained only two

Figure 3. Immunogold labeling of filaments from AD (A) and Pick's disease (B, C, and D) using antibodies PHF-1 (A and B), Tau 14 (C), and Tau 46.1 (D). Scale bar, 100 nm.

A

B

polypeptides of 55 kd and 64 kd that stained with PHF-1 (Figure 4B). The PHF-1-banding patterns were similar to those previously reported in brain homogenates using antibodies to other phosphorylated epitopes.^{24,25}

To further characterize Pick's filaments, immunoblots of polymer-enriched fractions were probed with a panel of phosphate-independent tau antibodies that included three exon-specific antibodies. With Tau 14 and Tau 46.1, four polypeptides of 55, 60, 64, and 68 kd were detected (Figure 4A). Two of the polypeptides (55 and 64 kd) had the same mobility as the PHF-1-positive polypeptides, suggesting that they contained tau phosphorylated at the PHF-1 site (Ser^{396/404}), whereas the 60- and 68-kd bands were not phosphorylated at this site. Probing with exon-specific antibodies indicated that Pick filament polypep-

Figure 4. Western blot analysis of filaments purified from Pick's disease cases. **A:** Filament-enriched fractions from AD and Pick's disease (PiD) were immunoblotted with PHF-1. Filament-enriched fractions from Pick's disease were also immunoblotted with the antibodies Tau 14 (T14) and Tau 46.1 (T46.1). Four bands (55, 60, 64, and 68 kd) were immunoreactive with Tau 14 and Tau 46.1 while only two bands (55 and 64 kd) were immunoreactive with PHF-1, suggesting that the PHF-1 epitope was phosphorylated in some but not all polypeptides ($n = 3$ Pick's cases). Exon-specific antibodies were used to immunoblot Pick's disease tau filaments. The exon 2-specific antibody (E2) and the exon 10-specific antibody (E10) labeled Pick's filaments, but the exon 3-specific antibody (E3) did not ($n = 3$). The specificity of the exon 10 antibody was tested using purified recombinant 2N4R tau and purified fetal tau. Although fetal tau did not immunolabel with the exon 10-specific antibody, it did label with antibody Tau 46.1. **B:** Filament-enriched fractions from AD and Pick's disease brain were dephosphorylated using alkaline phosphatase before SDS-PAGE and immunoblotted with the Tau-1 antibody. Tau protein standards indicated on the left are (from top to bottom) 2N4R, 2N3R, 1N4R, 1N3R, 0N4R, and 0N3R. The filament-enriched fraction from AD indicated all six isoforms, although the 2N4R and 2N3R bands were not a major species. Slight differences exist between the two Pick's cases analyzed, with PiD1 indicating the 1N3R, 0N4R, and 0N3R isoforms and PiD2 indicating the 2N3R, 1N3R, and 0N3R isoforms. These results were confirmed using the exon-specific antibodies (data not shown).

Figure 5. Filaments purified from AD brain (A), and Pick's disease brain (B, C, and D) were analyzed using STEM. Pick's filaments were both straight (B) and PHF-like (C) filaments. Scale bar, 100 nm.

tides are composed of tau isoforms expressing exons 2 and 10, but not exon 3 (Figure 4A). Because the presence of exon 10 in Pick's filaments was unexpected,^{24,25} the specificity of exon 10-specific antibody was verified against 2N4R recombinant tau, an isoform that expresses this exon (Figure 4) and against fetal tau, which does not (Figure 4). A similar verification was performed with antibodies specific to exons 2 and 3 by blotting them against recombinant tau isoforms either containing or lacking these exons (data not shown). A final verification was performed using filament-enriched fractions from AD and Pick's disease brain that were treated with alkaline phosphatase before SDS-PAGE, and immunoblotted with the Tau-1 antibody (Figure 4B) and the exon-specific antibodies (data not shown). These results confirmed the presence of both 4R and 3R tau isoforms in the filaments derived from Pick's disease brain, although slight differences in the isoform composition exist for the two cases shown.

STEM Analysis of Filaments

The physical parameters of Pick's filaments, AD filaments, and filaments assembled *in vitro* were examined using STEM. The mass/nm length, density, width, period, and appearance of AD-PHF's measured here (Figure 5A, Table 1) were similar to previously reported results.⁴⁰ The Pick's filaments were characterized by morphology as single or double filaments. Single filaments (Figure 5B) have a mass/nm length of ~72 kd/nm length and a density identical to that of AD-PHF's (Table 1). Double filaments included those that were fused PHF's displaying obvious periodicity (Figure 5C) and those that were clearly SF's lying next to each other (Figure 5D). All three types of Pick's filaments were the same densities as AD-PHF's (Table 1). The filaments shown in Figure 5D demonstrate two properties of the Pick's filaments. First,

two filaments lying next to each other have a mass/nm length that is identical to that of truly paired filaments (Figure 5C). Second, although the unstained STEM samples have low resolution, the filaments of Figure 5D also appear to cross over at two points with a period of 110 nm, perhaps indicative of an early pairing event (see below). A similar cross-over event was observed in Figure 2B.

The structure of Pick's filaments was also compared to that of filaments assembled from individual recombinant human tau isoforms and to those assembled from purified bovine brain tau that contain a mixture of all six isoforms (Figure 6). In our paradigm, only isoforms containing at least one N-terminal exon (E2 and E2/E3) assemble into filaments.⁴³ Therefore, the recombinant tau filaments studied were assembled from the remaining four isoforms (Figure 6A), primarily producing single, unpaired polymers (Figure 6; B-D, F, and H). The mass per unit length of these filaments ranged from 56 to 77 kd/nm length (Table 2) and except for 1N3R and 1N4R filaments was identical to that of the single filaments of Pick's disease. In some *in vitro*-assembled preparations (Figure 6; E, G, and I), double filaments were also apparent, as identified based on morphology, width, and mass/nm length. Double filaments were not found in assembled preparations from bovine tau and the 1N4R isoform. The mass/nm length of double filaments was very similar to that of AD-PHF's and Pick's PHF's (Table 1 and Table 2).

In comparison, the density of single isoform-assembled filaments, whether in single or double morphology or made of 3R or 4Rtau isoforms, was significantly lower than that of AD-PHF's and Pick's disease-derived filaments (Table 1 and Table 2). This could be because of the fact that the *in vitro* assembled filaments were often wider or had less mass/nm length than the *in vivo* filaments. Finally, the mass/nm length and density of *in vitro* assembled bovine filaments was not significantly differ-

Table 1. STEM Analysis of Filament-Enriched Fraction of AD and Pick's Disease Brain

	Mass/Length (kd/nm)	Density (kd/nm ³)	Width (nm)	Period (nm)	N
AD-PHF	114.90 ± 27.88	0.50 ± 0.12	15.19 ± 2.82	76.12 ± 11.87	27
Pick's disease					
Single	71.53 ± 10.29	0.49 ± 0.07*	12.05 ± 2.14	N/A	29
Double	113.65 ± 26.92*	0.44 ± 0.10*	16.07 ± 4.42	117.85 ± 35.94†	28

Mass/nm length, density, width, and period were calculated as in Methods.

*Not significant compared to AD-PHF's.

†Average of Peaks 1 and 2 as seen in Figure 2. Data shown ± SD.

Figure 6. cDNA constructs of the represented tau isoforms (A) were used to assemble filaments *in vitro*. STEM analysis of *in vitro* assembled filaments from whole bovine brain tau (C), and the isoforms 1N4R (B), 1N3R (D and E), 2N3R (F and G), and 2N4R (H and I) TMV filament. Single filaments (B–D, F, and H) and double filaments (E, G, and I) were observed. Bar = 100 nm for all panels. See Materials and Methods for definitions of single and double filaments.

ent from that of AD- PHFs or Pick's disease filaments (Table 1 and Table 2) (see Discussion).

Pairing of Filaments

It has been suggested that the filaments of Pick's disease do not display the classic PHF morphology because they represent a transitional stage in filament morphology; that is, they are progressing slowly from SFs to PHF-like filaments during the disease process, thus explaining the presence of long periodicities.¹² Such an hypothesis has also been advanced to explain why neuropil threads in AD are composed primarily of SFs.⁸ Moreover, recent *in vitro* experiments have demonstrated that prolonged incubation of recombinant tau in the presence of arachidonic acid leads to a progression of SFs to PHFs.^{43,46}

These hypotheses are partially supported by STEM analysis of pairing *in vitro*-assembled tau filaments. Single isoform filaments have been observed to twist together in a forked appearance after as little as 8 hours of assembly at room temperature (Figure 7A). A similar filament was examined by STEM (Figure 7C); each arm of the pairing filament is 65 kd/nm, and the portion twisted together has a mass/nm length of 120 kd/nm. Thus, the mass/nm length of a double filament can reflect the presence of two distinct filaments intertwined. Separate filaments of an AD-PHF can be distinguished; the addition of recombinant tau protein to purified AD-PHFs in the presence of arachidonic acid results in two SFs emanating from the original PHF (Figure 7B). Additionally, the paired filaments of Pick's disease filaments often resolve into two separate filaments (Figure 7D), exhibiting mass values (125 kd/nm) and density (0.49 kd/nm³) very similar to those obtained from *in vitro*-assembled double filaments (Table 2) as well as AD and Pick's PHFs (Table 1). Thus, both morphological and structural findings further support the contention that a PHF is comprised of two separate filaments intertwined.

Discussion

Pick's Versus AD Filaments

The most remarkable finding of this study is that isolated Pick's filaments contain both 3R (exon 10-deficient) and 4R (exon 10-containing) tau isoforms. This result is in contrast to previously published reports on Pick's disease^{24,25} that suggest the exclusive presence of 3R tau in Pick's filaments. Because the same exon-10-specific antibody was used in both laboratories, the different results obtained are most likely because of the fact that the previous studies analyzed whole brain homogenates. Such preparations contain both filamentous and nonfilamentous tau protein, whereas the filament-enriched fractions used in our study lack soluble tau. The predominance of 3R tau in the total homogenates from Pick's brains may well be because of sequestration of much of the 4R tau into less soluble Pick bodies. Alternatively, if Pick's brains produce primarily 3R tau as has been reported,^{24,25} then the neurons displaying the fibrillar pa-

Table 2. STEM Analysis of Filaments Synthesized in the Presence of Arachidonic Acid and Dithiothreitol

	Single filament				Double filament			
	Mass/Length (kD/nm)	Density (kD/nm ³)	Width (nm)	N	Mass/Length (kD/nm)	Density (kD/nm ³)	Width (nm)	N
Bovine	77.15 ± 19.99	0.44 ± 0.05 [†]	12.82 ± 2.47	76	N/A	N/A	N/A	
1N3R	59.14 ± 15.20	0.41 ± 0.10*	12.06 ± 2.06	19	100.60 ± 0.46	0.40 ± 0.00 [‡]	15.10 ± 3.18	2
2N3R	75.62 ± 9.52	0.33 ± 0.04*	15.19 ± 2.52	28	106.72 ± 10.61	0.29 ± 0.03*	19.09 ± 3.88	11
1N4R	55.90 ± 12.70	0.34 ± 0.08*	12.85 ± 2.17	15	N/A	N/A	N/A	
2N4R	74.55 ± 17.45	0.37 ± 0.09*	14.02 ± 2.09	89	110.53 ± 10.75	0.31 ± 0.03*	19.03 ± 2.07	9

Values are means ± SD (or range) for the *n* number of examined filaments. Samples were analyzed after 24 hours of incubation at room temperature. The density of single isoform but not bovine filaments significantly differed from that of AD-PHF and Pick's disease derived filaments.

N/A, data not available (double filaments absent).

*P < 0.001.

[†]NS as compared to respective filaments in AD and Pick's disease (see Table 1).

[‡]Only two filaments were found.

thology must represent a subpopulation expressing predominantly 4R tau isoforms.

The results of the present study strongly suggest that the presence of hyperphosphorylated tau isoforms alone may prove ambiguous in differentiating filamentous and soluble tau pools. For example, we have found that only a fraction of the isoforms comprising Pick's filaments are phosphorylated at the PHF-1 site (phospho-Ser^{396/404}). The reason for this partial phosphorylation of the tau isoforms in Pick's disease is unknown, and it stands in marked contrast to what is observed in AD, in which all six tau isoforms display the PHF-1 phosphoepitope.⁴⁷ Perhaps the enriched filament fraction from Pick's brain is composed of polymers derived from various subcellular compartments in which the kinase/phosphatase activities are different. In this regard, it will be instructive to assay

for other phosphoepitopes in Pick's filaments and to determine the ratio of individual phosphorylated *versus* non-phosphorylated tau species. Such comparisons may help identify phosphorylation sites that are crucial to the assembly process as opposed to those that are introduced after tau polymers form.⁴⁸

In AD, tau protein undergoes aggregation into two morphologically distinct types of filaments, PHFs and SFs. In the present study, we have demonstrated that the Pick's filaments display three morphological types: SFs, PHFs with long periodicities, and AD-like PHFs. Despite the multiple filament types observed and striking differences in pathological inclusions that characterize these two diseases, the mass/nm length and density in Pick's filaments were identical to those found in AD-PHF.

Figure 7. Electron micrographs of assembled preparations of isoform 2N4R after 8 hours of incubation in the presence of arachidonic acid (A) indicates a possible early pairing event. The addition of 2N4R to AD-PHF results in growth off one end of the PHF (B). By STEM, 2N4R (C) shows a similar morphology, with each arm of the fork having a mass/nm length of 65 kd, and the pairing region exhibits a mass/nm length of 120 kd. Filaments of Pick's disease (D) also show a clear distinction between each hemi-filament of the PHF. Scale bars, 100 nm.

STEM Analysis of *In Vitro*-Assembled Filaments

A previous comparison between the packing density of tau within the filaments of CBD and AD demonstrated that CBD filaments were significantly less dense.⁴⁹ Interestingly, CBD filaments contain exclusively 4R tau isoforms, whereas both AD and Pick's filaments exhibit the presence of one or more 3R isoforms in addition to 4R tau isoforms. This raises the possibility that the tau subunit packing density may depend on the ratio of 3R to 4R tau isoforms in the filament lattice. Although no difference in packing density was observed between 3R and 4R tau filaments assembled *in vitro*, the single isoform filament densities determined were very similar to those found in CBD,⁴⁹ but significantly different from those found in AD or Pick's disease. Furthermore, *in vitro*-assembled bovine tau filaments, containing a mixture of all six CNS tau isoforms, were equal in density to AD and Pick's filaments. These results suggest that the packing density of tau within filaments may be determined, in part, by interactions of the microtubule-binding repeats. Therefore, filaments containing both 4R tau and 3R tau may exhibit a more compact arrangement of subunits and/or protofilaments than those assembled from 4R tau or 3R tau alone. In this regard, it will be instructive to determine the mass/nm length and density of filaments from PSP and some of the FTDP-17 cases characterized by 4R-only tau filaments.

The Relationship of SF and PHF

The relationship between PHF and SF has been a hotly debated topic for years. PHFs are generally considered the primary filamentous morphology in AD.⁷ However, SFs are the most abundant type of filaments within AD neuropil threads,⁸ and are prominent in many of the tauopathies.¹⁸ The relationship between these two morphological representations of tau filaments is becoming clearer. Previously we have shown that the incubation of recombinant tau protein in the presence of arachidonic acid results in the formation of mostly SF, but that after a number of days these SFs appear to twist together and form PHF-like structures.^{43,46} The STEM analyses further support these findings, demonstrating clearly that each arm of a forming PHF-like filament has a mass/nm length that is approximately half that of the twisted region. Furthermore, Pick's disease filament populations seem to exhibit morphologies similar to those observed *in vitro*, suggesting that PHF formation is a stepwise process. Specifically, SF formation is followed by pairing and fusion along the axes of the individual filaments. With time, these paired filaments may become twisted displaying progressively shorter periodicities, finally forming PHFs.

Conclusions

In summary, filaments purified from Pick's disease are similar to those of AD in their antigenic character, mass/nm length, width, and density. They do not seem to be composed of all six tau isoforms, but contain two 4R isoforms and one or two 3R isoforms. Morphologically, mostly SFs are observed, but paired filaments with long periodicities, and those with a period similar to that of AD-PHF, also are present, suggesting a progression from SF to PHF. Finally, *in vitro*-assembled single isoform filaments are similar in mass/nm length to those found in AD and Pick's disease, but they are less dense, resembling more closely filaments found in the brains of patients with CBD.

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References

1. Komori T: Tau-positive glial inclusions in progressive supranuclear palsy, corticobasal degeneration and Pick's disease. *Brain Pathol* 1999, 9:663-679

2. Braak H, Braak E: Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol* 1991, 82:239-259
3. Braak E, Arai K, Braak H: Cerebellar involvement in Pick's disease: affliction of mossy fibers, monodendritic brush cells, and dentate projection neurons. *Exp Neurol* 1999, 159:153-163
4. Braak H, Braak E: Involvement of precerebellar nuclei in Pick's disease. *Exp Neurol* 1998, 153:351-365
5. Braak E, Braak H, Mandelkow EM: A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads. *Acta Neuropathol* 1994, 87:554-567
6. Wischik CM, Novak M, Edwards PC, Klug A, Tichelaar W, Crowther RA: Structural characterization of the core of the paired helical filament of Alzheimer disease. *Proc Natl Acad Sci USA* 1988, 85:4884-4888
7. Wischik CM, Crowther RA, Stewart M, Roth M: Subunit structure of paired helical filaments in Alzheimer's disease. *J Cell Biol* 1985, 100:1905-1912
8. Perry G, Mulvihill P, Manetto V, Autilio-Gambetti L, Gambetti P: Immunocytochemical properties of Alzheimer straight filaments. *J Neurosci* 1987, 7:3736-3738
9. Jellinger K, Danielczyk W, Fischer P, Gabriel E: Clinicopathological analysis of dementia disorders in the elderly. *J Neurol Sci* 1990, 95:239-258
10. Takauchi S, Hosomi M, Marasigan S, Sato M, Hayashi S, Miyoshi K: An ultrastructural study of Pick bodies. *Acta Neuropathol* 1984, 64:344-348
11. Sparkman DR, Johnson SA, Hammon KM, Allison PM, White CLD: Isolation of the insoluble straight fibrils of Pick's disease. *J Neurol Sci* 1987, 80:173-184
12. Kato S, Nakamura H: Presence of two different fibril subtypes in the Pick body: an immunoelectron microscopic study. *Acta Neuropathol* 1990, 81:125-129
13. Murayama S, Mori H, Ihara Y, Tomonaga M: Immunocytochemical and ultrastructural studies of Pick's disease. *Ann Neurol* 1990, 27:394-405
14. Armstrong RA, Cairns NJ, Lantos PL: Quantification of pathological lesions in the frontal and temporal lobe of ten patients diagnosed with Pick's disease. *Acta Neuropathol (Berl)* 1999, 97:456-462
15. Himmler A: Structure of the bovine tau gene: alternatively spliced transcripts generate a protein family. *Mol Cell Biol* 1989, 9:1389-1396
16. Goedert M, Spillantini MG, Potier MC, Ulrich J, Crowther RA: Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain. *EMBO J* 1989, 8:393-399
17. Goedert M, Spillantini MG, Jakes R, Crowther RA, Vanmechelen E, Probst A, Gotz J, Burki K, Cohen P: Molecular dissection of the paired helical filament. *Neurobiol Aging* 1995, 16:325-334
18. Buee L, Delacourte A: Comparative biochemistry of tau in progressive supranuclear palsy, corticobasal degeneration, FTDP-17 and Pick's disease. *Brain Pathol* 1999, 9:681-693
19. Hutton M, Lendon CL, Rizzu P, Baker M, Froelich S, Houlden H, Pickering-Brown S, Chakraverty S, Isaacs A, Grover A, and others: Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature* 1998, 393:702-705
20. Spillantini MG, Crowther RA, Kamphorst W, Heutink P, van Swieten JC: Tau pathology in two Dutch families with mutations in the microtubule-binding region of tau. *Am J Pathol* 1998, 153:1359-1363
21. Poorkaj P, Bird TD, Wijsman E, Nemens E, Garruto RM, Anderson L, Andreadis A, Wiederholt WC, Raskind M, Schellenberg GD: Tau is a candidate gene for chromosome 17 frontotemporal dementia. *Ann Neurol* 1998, 43:815-825
22. Hasegawa M, Smith MJ, Iijima M, Tabira T, Goedert M: FTDP-17 mutations N279K and S305N in tau produce increased splicing of exon 10. *FEBS Lett* 1999, 443:93-96
23. D'Souza I, Poorkaj P, Hong M, Nochlin D, Lee VM, Bird TD, Schellenberg GD: Missense and silent tau gene mutations cause frontotemporal dementia with Parkinsonism-chromosome 17 type, by affecting multiple alternative RNA splicing regulatory elements. *Proc Natl Acad Sci USA* 1999, 96:5598-5603
24. Delacourte A, Robitaille Y, Sergeant N, Buee L, Hof PR, Watzet A, Laroche-Chollette A, Mathieu J, Chagnon P, Gauvreau D: Specific pathological Tau protein variants characterize Pick's disease. *J Neuropathol Exp Neurol* 1996, 55:159-168

25. Delacourte A, Sergeant N, Wattez A, Gauvreau D, Robitaille Y: Vulnerable neuronal subsets in Alzheimer's and Pick's disease are distinguished by their tau isoform distribution and phosphorylation. *Ann Neurol* 1998, 43:193-204
26. Murrell JR, Spillantini MG, Zolo P, Guazzelli M, Smith MJ, Hasegawa M, Redi F, Crowther RA, Pietrini P, Ghetti B, and others: Tau gene mutation G389R causes a tauopathy with abundant pick body-like inclusions and axonal deposits. *J Neuropathol Exp Neurol* 1999, 58:1207-1226
27. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L: The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* 1991, 41:479-486
28. Mirra SS, Hart MN, Terry RD: Making the diagnosis of Alzheimer's disease. A primer for practicing pathologists [see comments]. *Arch Pathol Lab Med* 1993, 117:132-144
29. Mirra SS: Neuropathological assessment of Alzheimer's disease: the experience of the Consortium to Establish a Registry for Alzheimer's Disease. *Int Psychogeriatr* 1997, 9(Suppl 1):S263-S272
30. Mirra SS: The CERAD neuropathology protocol and consensus recommendations for the postmortem diagnosis of Alzheimer's disease: a commentary. *Neurobiol Aging* 1997, 18(4 Suppl):S91-S94
31. Munoz-Garcia D, Ludwin SK: Classic and generalized variants of Pick's disease: a clinicopathological, ultrastructural, and immunocytochemical comparative study. *Ann Neurol* 1984, 16:467-480
32. Dickson DW, Ksiezak-Reding H, Davies P, Yen SH: A monoclonal antibody that recognizes a phosphorylated epitope in Alzheimer neurofibrillary tangles, neurofilaments and tau proteins immunostains granulovacuolar degeneration. *Acta Neuropathol* 1987, 73:254-258
33. Kosik KS, Orecchio LD, Binder L, Trojanowski JQ, Lee VM, Lee G: Epitopes that span the tau molecule are shared with paired helical filaments. *Neuron* 1988, 1:817-825
34. Carmel G, Mager EM, Binder LI, Kuret J: The structural basis of monoclonal antibody Alz50's selectivity for Alzheimer's disease pathology. *J Biol Chem* 1996, 271:32789-32795
35. Greenberg SG, Davies P, Schein JD, Binder LI: Hydrofluoric acid-treated tau PHF proteins display the same biochemical properties as normal tau. *J Biol Chem* 1992, 267:564-569
36. Biernat J, Mandelkow EM, Schroter C, Lichtenberg-Kraag B, Steiner B, Berling B, Meyer H, Mercken M, Vandermeeren A, Goedert M, and others: the switch of tau protein to an Alzheimer-like state includes the phosphorylation of two serine-proline motifs upstream of the microtubule binding region. *EMBO J* 1992, 11:1593-1597
37. Yang LS, Gordon-Krajcer W, Ksiezak-Reding H: Tau released from paired helical filaments with formic acid or guanidine is susceptible to calpain-mediated proteolysis. *J Neurochem* 1997, 69:1548-1558
38. Ghoshal N, Smiley JF, DeMaggio AJ, Hoekstra MF, Cochran EJ, Binder LI, Kuret J: A new molecular link between the fibrillar and granulovacuolar lesions of Alzheimer's disease. *Am J Pathol* 1999, 155:1163-1172
39. Yang L, Ksiezak-Reding H: Ubiquitin immunoreactivity of paired helical filaments differs in Alzheimer's disease and corticobasal degeneration [In Process Citation]. *Acta Neuropathol (Berl)* 1998, 96:520-526
40. Ksiezak-Reding H, Wall JS: Mass and physical dimensions of two distinct populations of paired helical filaments. *Neurobiol Aging* 1994, 15:11-19
41. Gordon-Krajcer W, Yang L, Ksiezak-Reding H: Conformation of paired helical filaments blocks dephosphorylation of epitopes shared with fetal tau except Ser199/202 and Ser202/Thr205. *Brain Res* 2000, 856:163-175
42. Wilson DM, Binder LI: Polymerization of microtubule-associated protein tau under near-physiological conditions. *J Biol Chem* 1995, 270:24306-24314
43. King ME, Gamblin TC, Kuret J, Binder LI: Differential assembly of human tau isoforms in the presence of arachidonic acid. *J Neurochem* 2000, 74:1749-1757
44. Ksiezak-Reding H, Yang G, Simon M, Wall JS: Assembled tau filaments differ from native paired helical filaments as determined by scanning transmission electron microscopy (STEM). *Brain Res* 1998, 814:86-98
45. Probst A, Tolnay M, Langui D, Goedert M, Spillantini MG: Pick's disease: hyperphosphorylated tau protein segregates to the somato-axonal compartment. *Acta Neuropathol (Berl)* 1996, 92:588-596
46. King ME, Ahuja V, Binder LI, Kuret J: Ligand-dependent tau filament formation: implications for Alzheimer's disease progression. *Biochemistry* 1999, 38:14851-14859
47. Delacourte A, Buee L: Normal and pathological tau proteins as factors for microtubule assembly. *Int Rev Cytol* 1997, 171:167-224
48. Schneider A, Biernat J, von Bergen M, Mandelkow E, Mandelkow EM: Phosphorylation that detaches tau protein from microtubules (Ser262, Ser214) also protects it against aggregation into Alzheimer paired helical filaments. *Biochemistry* 1999, 38:3549-3558
49. Ksiezak-Reding H, Tracz E, Yang LS, Dickson DW, Simon M, Wall JS: Ultrastructural instability of paired helical filaments from corticobasal degeneration as examined by scanning transmission electron microscopy. *Am J Pathol* 1996, 149:639-651